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Bactrocera latifrons in Europe: the importance of the right attractant for detection

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Abstract

After the first finding of *Bactrocera dorsalis* (Hendel) in Italy, an emergency monitory plan was adopted in the infested area aimed at confirming or denying the establishment of this species in Italy. Scheduling the installation of traps set with both Methyl eugenol and Torula yeast, in 2019 the monitory plan allowed other captures of *B. dorsalis* and captures of a new Tephritidae, *Bactrocera latifrons* (Hendel), arousing large concern. Also known as solanum fruit fly, *B. latifrons*, although shows a smaller host range than other conspecific entities, could be particularly damaging in the temperate climate where nightshades and cucurbits represent major crops. An integrative characterization (morphological and molecular) was carried out, showing the finding of two different COI haplotypes which origin could not be confirmed. In 2020 monitoring activities continued, but no specimens of both the species were captured, presumably due to the temporary cessation in travels and trade during the pandemic emergency. Results highlighted the importance of using different attractants to increase the chances of intercept a larger number of invasive pests and promptly lay out a monitoring and control program.

Key words: first record, fruit fly, invasive insect, male lure, solanum fruit fly, SFF, torula yeast, traps.

Introduction

In the last decades, exotic species invasion has increased across the world (Seebens *et al*., 2017). The main factors influencing this trend are unquestionably the movements of people and vegetable materials, which simplify, as never before, the accidental introduction of species in new areas (Seebens *et al*., 2015; Chapman *et al*., 2017). Italy and other countries around the Mediterranean basin are susceptible to exotic species establishment due to the warm-temperate climate, made even warmer by the occurring climate changes (Lupi *et al*., 2014; Lionello and Scarascia, 2018; Nugnes *et al*., 2020; Pyšek *et al*., 2020). The tropical and subtropical fruit flies (Diptera Tephritidae) are considered among the most dangerous pests in agriculture because of their elevated biotic and invasive potential in temperate areas (Papadopoulos, 2014; Mcinnis *et al*., 2017).

In Europe, several Tephritidae species are regulated through the Implementing Regulation 2019/2072 (which replaces the European Directive 29/2000/EC), and even more are present in the European Plant Protection Organization (EPPO) lists (A1 and A2) (EPPO, 2021a; 2021b).

The genus *Bactrocera* Macquart (Diptera Tephritidae) comprises more than 600 species (Yaakop *et al*., 2013) and about 70 of them constitute a threat to agriculture worldwide (Shelly, 2010). Currently, four *Bactrocera* species or complexes of species are listed in the Implementing Regulation 2019/2072. These are: the species-complex *Bactrocera dorsalis* (Hendel) (the Oriental fruit fly), the species-complex *Bactrocera tryoni* (Froggatt) (the Queensland fruit fly), the species *Bactrocera zonata* (Saunders) (the peach fruit fly), and *Bactrocera tsuneonis* (Miyake) (the Japanese orange fly).

While *B. tryoni* and *B. tsuneonis* are slowly enlarging their distribution within the native continents (Oceania and Asia), *B. zonata* and *B. dorsalis*, indigenous to Southeast Asia and Asia respectively, in last years have hugely expanded their distribution. At present, *B. zonata* distribution area includes Asia, Middle East, and several African countries, while *B. dorsalis* is widely distributed in South-Southeast Asia, in almost all African countries, on the Hawaiian Islands, and besides, it is often intercepted in the USA mainland (Steck *et al*., 2019). The ongoing invasive processes of *B. dorsalis* and *B. zonata* are increasing the alert level in the Mediterranean region (Lux *et al*., 2003; Mahmoud *et al*., 2020).

Bactrocera latifrons (Hendel), the solanum fruit fly (SFF), is another harmful species showing high biological potential recorded in the EPPO A1 list, whose distribution is widening (Mwatawala *et al*., 2007). *B. latifrons* is a multivoltine species that, in suitable conditions, can complete a cycle (egg-adult) in about 20 days and females can lay up to 600 eggs in ripening fruits in their lifespan (Vargas and Nishida, 1985). Besides, this fruit fly can probably establish in temperate and tropical climates (Liquido *et al*., 1994). *B. latifrons* is a polyphagous species, but it has a host plant range smaller than the aforementioned species. *B. latifrons* damages mainly nightshades (Solanaceae), preferring the species of the genus *Capsicum* sp. characterized by softer fruits with lower phenolic content (Rattanapun *et al*., 2021), only occasionally it can attack cucurbits (Cucurbitaceae), and rarely other plant families (Liquido *et al*., 1994; Paulsen, 2007; Mziray *et al*., 2010). Several studies also highlighted the probability that *B. latifrons* may survive outside managed crop fields, due to its ability to develop on the wild plant too, like the cosmopolitan species *Solanum nigrum* L. (Liquido *et al*., 1994; Mziray *et al*., 2010; McQuate and Liquido, 2013).

Given the high invasive potential and the risk of accidental introductions, the early detection of fruit fly specimens on the territory is mandatory, while preventive monitoring is probably the best way to avoid the high economic costs of late eradication plans (Martinez *et al*., 2020). Several studies showed how the employment of trap and specific lure is a valuable tool to detect incursions since possible early establishment (Mcinnis *et al*., 2017). These substances act as baits for males belonging to the subfamily Dacinae, tribe Dacini, which includes the genera *Bactrocera*, *Dacus, Monacrostichus*, and *Zeugodacus* (Manrakhan *et al*., 2017; McQuate *et al*., 2018). The *Bactrocera* species can be divided into three major groups based on the kind of male lure that is effective on males: species attracted by methyl-eugenol (ME), species attracted by Cue-Lure/Raspberry ketone (CL/RK), or species non-responsive, that are not attracted either by ME or CL/RK (Shelly, 2010; Tan *et al*., 2014; McQuate *et al*., 2018). Despite ME is strongly effective on males of about 80 *Bactrocera* species, included *B. dorsalis* and *B. zonata* (Tan and Nishida, 2012; FAO/IAEA, 2018), the group of non-responsive species includes important pests too, such as *B. latifrons* (McQuate *et al*., 2018). The last one however shows attraction from several substances containing α-ionol or α-Ionone (Mcgovern *et al*., 1989), and the effect of these chemicals is reinforced when they are combined with phenolic volatiles, especially Cade Oil, an essential oil extracted from twigs of *Juniperus oxycedrus* L. (McQuate and Peck, 2001; McQuate *et al*., 2004; 2018).

To accomplish the European Directive 2000/29/EC, a *Bactrocera* spp. surveillance monitoring program started in Campania region (Italy) in 2018. This monitoring has been carried out employing 10 McPhail traps baited with ME and led to the first records in the field of specimens of *B. dorsalis* in Europe (Nugnes *et al*., 2018).

The intense monitoring plan immediately following this event had scheduled the installation of about two thousand McPhail traps in the same finding areas. In the monitory plan both ME and protein baits were employed (Vitiello *et al*., 2020) because the need of tephritid females to get proteins to improve oocytes and egg production makes the last one highly attractive to females (Fontellas and Zucoloto, 1999; Aluja *et al*., 2001; Epsky, 2014). The use of protein baits constituted by torula yeast (TY) and water (Epsky, 2014; Shelly *et al*., 2020) allows also to widen the spectrum of monitored species, including other harmful tephritids that are non-responsive to ME.

The aims of this work were to verify the presence of exotic tephritid species using different attractants and, in the case of finding, to assess whether their presence is to be considered a simple incursion or a real establishment.

Materials and methods

Monitoring plan 2019

In 2019, following the records of seven *B. dorsalis* specimens in two Campanian sites in 2018 (Nugnes *et al*., 2018), an emergency monitory plan was adopted. The monitoring plan, based on the "trapping guidelines for area-wide fruit fly programmes" (FAO/IAEA, 2018), was scheduled and adopted as described in Nugnes *et al*. (2019) and Vitiello *et al*. (2020), employing in total about 1732 traps in 64 km^2 around the record sites.

Two circular areas, called "delimited areas" (1 = Palma Campania; 2 = Nocera Inferiore) with the centre at the site of the capture and diameter of 8 km, were individuated on the map. These circles were slightly overlapped and further subdivided into four concentric areas: reinforced area A, closer to the capture site (with 40 traps per square km), reinforced area B (20 traps/km^2), reinforced area C (10 traps/km^2), reinforced area D considered as buffer area (1 trap/km^2), for a total of 2034 traps (figure 1). Due to the geomorphology, the percentage of urbanisation of the areas and the overlapping of the two areas, a reduction of about 20% of the total was planned, and the final scheduled number of traps was 1627.

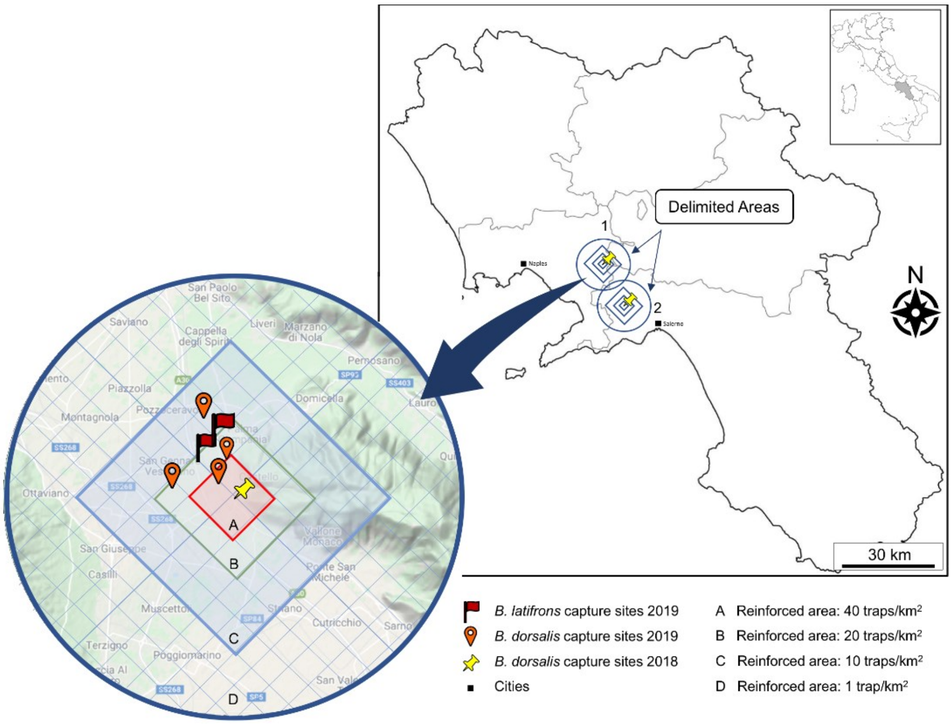


Figure 1. Map with detail of the monitored areas and captures.

The placement of the 1627 traps (McPhail) has been done in sites chosen for: 1) the presence of *B. dorsalis* major and minor host plants, such as fruit trees (*Prunus* spp., *Citrus* spp., *Diospyros kaki*) and crops (*Solanum lycopersicum*, *Solanum melongena*, *Capsicum* spp., *Cucurbita* spp.); 2) the absence or low use of chemical treatments. Due to the dense urbanization of the areas, many sites were suburban or private gardens, with few orchards or small farmers and even some abandoned fields.

Among the 1627 traps, almost all (1611 traps) were baited with ME dispensers (Tan *et al*., 2014; FAO/IAEA, 2018), replaced every four weeks, as indicated by the provider.

The remaining 16 traps (two per square km in A area) were baited with a TY solution (dry yeast/water - 1 gr/100 ml). About 250 ml of TY solution was poured into the lower part of the McPhail trap and replaced weekly. This kind of attractant was used initially only in 16 traps both because it is non-specific for *Bactrocera* species and because the traps with this attractant require more time than traps baited with ME to be prepared, replaced and controlled.

These numbers changed throughout the study: in fact, when in autumn 2019 some captures of *Bactrocera* spp. specimens occurred, the number of traps was increased, with the installation, in total, of 73 new traps with ME and 32 new traps with TY solution, spread around the finding sites. Thus, the final number of traps installed was 1732 (1684 McPhail with ME and 48 McPhail with TY).

Monitoring plan 2020

In 2020, continuing the monitoring of *B. dorsalis* in the same area, 400 McPhail traps baited with ME were installed. Among these, 20 traps were baited with TY solution.

Moreover, based on the captures of some fruit fly specimens in 2019, in the following year (2020), six traps baited with a mixture of Cade Oil and α-ionol, were used. In each trap, a circular sponge (⌀ 5 cm) was imbued with 4 ml of the mixture, previously made in laboratory combining 2 ml of Cade Oil and 2 ml of α-ionol. The sponge was replaced weekly (McQuate *et al*., 2004).

Sampling

All specimens caught in the McPhail traps baited with ME were easily collected using an entomological tweezer and stored in Petri dishes in the fridge.

The TY solution of McPhail traps was firstly rinsed with fresh water in a plastic box. Then, all the captured specimens were collected with a strainer and put in one or more Petri dishes. Subsequently, the samples, containing a large number of others flies (from 10 to 300), were taken to the laboratory to perform a deeper investigation. Using a stereoscope, a first selection was made by collecting only the Tephritidae specimens, then any individuals belonging to the genus *Bactrocera* were isolated and separated from the other flies.

Integrative characterization

All the exotic fruit flies caught during the two years of sampling were subjected to an integrative characterization.

The target specimens belonging to *Bactrocera* genus were initially identified using the available taxonomic keys and description (Drew and Hancock, 1994; Mahmood, 2004; Drew *et al*., 2005; David and Ramani, 2012; Plant Health Australia, 2018). Subsequently, a molecular analysis was performed as in Nugnes *et al*. (2018). A metathoracic leg (tibia and tarsus) was separated with sterile tweezers from each captured specimen and used for total genomic DNA extraction with a Chelex and proteinase K protocol. Amplification reactions were performed on the extracted DNA to obtain a portion of the cytochrome c oxidase subunit I (COI) and, when necessary, the ribosomal ITS1 region. These portions have proved to be useful in the discrimination of the *Bactrocera* species (Boykin *et al*., 2014; Nugnes *et al*., 2018). Two primer pairs were used to obtain a COI portion of ~1400 bp (hereafter COI-AB): LCO-1490/HCO-2198 (Folmer *et al*., 1994), for a portion of ~640 bp (COI-A) and C1-J-2183/TL2-N-3014 (Simon *et al*., 1994), for a portion of ~810 bp (COI-B). ITS1 marker was amplified with primer pair ITS7-ITS6 with the conditions reported in Boykin *et al*. (2014).

The check of the PCR products was carried out with electrophoresis on a 1.2% agarose gel containing GelRED® and, after the direct sequencing, chromatograms were compared using BioEdit 7.0 (Hall, 1999) and manually edited. COI sequences were virtually translated into amino acids to detect nonsense codons or frameshift mutations using EMBOSS Transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Sequences of ITS1 were checked against the GenBank database by Blast searches (http://www.ncbi.nlm.nih.gov/BLAST) (last access July 1, 2021) and deposited in GenBank with accession numbers (MZ648334 - MZ648336).

Due to the discrepancy in homologous COI sequences in genetic databases (several barcoding, 3' region, and much less total COI sequences), the obtained sequences (COI-A, COI-B, and COI-AB) were checked separately against GenBank and matched against BOLD database through BOLD Identification System (http://www.boldsystems.org) (last access July 1, 2021).

The relationships among the collected samples and other *Bactrocera* species were evaluated. The species distances were estimated through Maximum likelihood analyses of COI-A and COI-B portions. Species included in analyses were selected based on searches performed by Blast, choosing a single sequence per species among the first 100 most similar sequences to our sequences of *B. latifrons*, and excluding the sequences of *B. latifrons* present in GenBank.

Furthermore, *B. latifrons* sequences farthest and nearest to sequences obtained in the present study were included. ML trees were performed with RaxMl 7.0.4 (Stamatakis, 2006). GTR+G+I evolutionary models, selected by jModeltest (Posada, 2008) were used for both COI datasets. 1000 rapid bootstrap pseudoreplicates were performed to support ML branches and trees were rooted using the midpoint-rooted tree option.

The intra-specific relationship among the collected samples and other *B. latifrons* specimens collected worldwide was performed too. All the homologous *B. latifrons* COI sequences, available in GenBank and

Table 1. Adults of *Bactrocera* spp. collected in McPhail traps in 2019. TY: torula yeast solution; ME: methyl-eugenol.

Site	Attractant	Locality	N. of caught fruit flies	Species	Date	Coordinates UTM (33T)	Sample code	Sex	Accession number
I	TY	Palma Campania	2	*B. latifrons*	02/10/2019	4524009N 461971E	BL_1 BL_2	♀ ♀	MZ621833 MZ621834
II	TY	Palma Campania	1	*B. latifrons*	30/10/2019	4524241N 462294E	BL_3	♀	MZ621835
III	ME	San Gennaro Vesuviano	1	*B. dorsalis*	03/09/2019	4522821N 460751E	BD_4	♂	MZ621836
IV	ME	Palma Campania	1	*B. dorsalis*	10/09/2019	4523852N 462516E	BD_5	♂	MZ621837
V	ME	Palma Campania	1	*B. dorsalis*	24/09/2019	4525494N 462059E	BD_6	♂	MZ621838
VI	ME	Palma Campania	1	*B. dorsalis*	15/10/2019	4523026N 462618E	BD_7	♂	MZ621839

BOLD, were aligned with our sequences for further analysis. Sequences from both databases were considered only once; hence, the number of total sequences was reduced as suggested by RAxML 7.0.4 (Stamatakis, 2006). Subsequently, sequences distances and standard errors were calculated with MEGA 6 software based on uncorrected *p*-distance. In detail, the minimum and the maximum distance between the Italian haplotypes and all the other sequences, the minimum and the maximum distance among all sequences were evaluated on three datasets relating to the considered portions. All values are presented with standard errors (± SE).

Because of the lack of available ITS1 sequences of samples conspecific to *B. latifrons* (BL) samples on both databases, this marker was excluded from the evaluation of the *p*-distances.

Results

On 2nd October 2019, two females of an exotic fruit fly - later determined as *B. latifrons* - were captured in a trap baited with TY solution in a site in Palma Campania (hereafter "site I"). A few weeks later, on 30th October, another female of the same species was captured in a different site in Palma Campania (hereafter "site II"), inside one of the additional 10 traps promptly installed within 400 m from the first capture (table 1). Site I is a small non-specialized private garden, with the presence of one *Ficus carica*, one *Prunus armeniaca* and, one *Punica granatum*, close to a small orchard with a few plants of *S. lycopersicum* and *S. melongena*. A fruit orchard with several *D. kaki* trees is located near the site I. Sheltered and bigger than site I, site II is a private garden with several *Citrus sinensis*, *Citrus reticulata* and *D. kaki* trees. Furthermore, in the neighbour fields about 40 plants of *S. lycopersicum*, *S. melongena*, *Capsicum annum*, and 10 plants of *Cucurbita pepo* were cultivated at the time of the findings.

The phenological stage of the possible host plants when the captures occurred were: the persimmon was fully ripe, citrus fruits were at veraison stage while few figs, tomatoes, eggplants, and peppers were still ripening on plants.

The monitoring with 1732 traps led also to the capture in September 2019 of four males of *B. dorsalis* specimens, with McPhail traps baited with ME only, in four different sites belonging to one of the delimited areas (Vitiello *et al*., 2020) (table 1).

During the monitoring carried out in 2020, the traps baited with ME, TY solution, and with the specific attractant Cade Oil/α-ionol did not capture any exotic fruit fly.

Integrative characterization

The 3 females captured in the TY solution in 2019 (identified as BL_1, BL_2 and BL_3) were morphologically identified as *B. latifrons* species (EPPO Code: DACULA) using the existing keys (Drew and Hancock, 1994; Mahmood, 2004; Drew *et al*., 2005; David and Ramani, 2012; Plant Health Australia, 2018). The effective morphological traits to identify *B. latifrons* females are the presence of yellowish vittae on the thorax, the apex of the costal band of the wing distinctly expended into a spot, and, the most useful character, the aculeus with trilobed apex (figure 2).

Molecular analysis of the 3 females confirmed morphological identification as *B. latifrons*, while the male samples caught in traps baited with ME were confirmed as *B. dorsalis.*

COI sequences highlighted BL_1 and BL_3 (captured in two different traps) were identical (hereafter will be named only BL_1), while BL_2 (captured together with BL_1) differed in 5 bps. These variations were found in COI-B portion, while COI-A was shared by all three specimens. Sequences of ITS1 obtained from the three studied samples were completely identical and no matches were found with *B. latifrons* in any databases due to the lack of *B. latifrons* sequences. The most similar sequence (91%) belonged to *Bactrocera psidii* (Froggatt).

Relationships among the collected samples and other *Bactrocera* species chosen for the analyses are reported in figure 3. In both cases *B. latifrons* samples resulted included in a distinct group along with the more distant and closest homologous conspecific sequences.

To evaluate the relationship among the collected specimens and other *B. latifrons* samples, three alignments

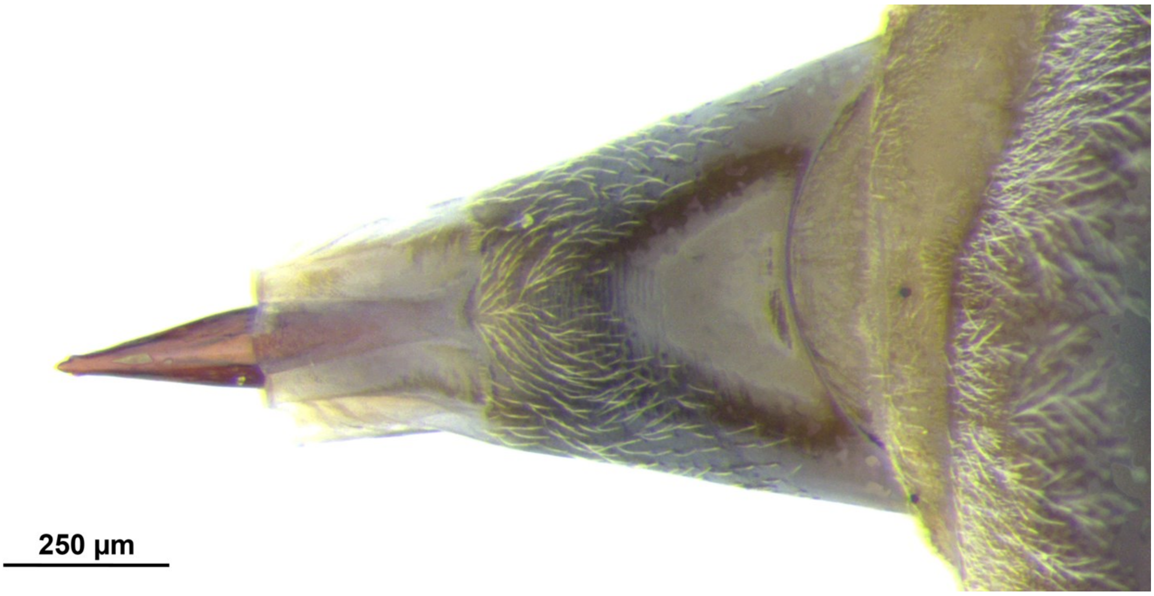


Figure 2. Particular of distinctive trilobate ovipositor of *B. latifrons*.

were obtained and compared: COI-A, COI-B and COI-AB. More than 100 sequences from GenBank and BOLD databases showed more than 99% of identity with COI-A, however, considering all the COI-A homologous sequences available in both databases, an alignment of 320 sequences was obtained. Thirty-four sequences resulted identical to the Italian COI-A, and they belong to insects collected in different part of the world: Thailand (n = 17), USA (Hawaii) (n = 6), China (Yunnan) (n = 4), Malaysia (n = 2), Vietnam (n = 1), no information (n = 4). The exclusion of identical sequences through RAxML led to a final alignment of 104 sequences used in the pairwise distance evaluation.

BL_1's COI-B portion resulted identical only to a Malaysian sequence (JX129505), while BL_2 was different from all the available sequences. COI-B dataset was reduced following the same method from 29 to 16 sequences, excluding the redundant sequences. COI-AB dataset did not show any identical sequence to Italian ones and, removing redundant results, was reduced from 24 to 15 sequences.

The four *B. dorsalis* COI sequences resulted highly similar among them, showing only from 4 to 7 different bps. The comparison with COI sequences from *B. dorsalis* males found in the same areas in 2018 (samples BD_1, BD_2, and BD_3 - 2018 - as reported in Nugnes *et al.*, 2018) highlighted that these new samples (BD_4-7 - 2019) are very similar to BD_3 - 2018 (from 1 to 7 different bps). All samples collected in 2019 are conversely quite dissimilar to the samples collected in 2018 BD_1/BD_2 with ~100 different bps in the COI sequences. Blast analyses resulted in similarity values >99% with homologous sequences of *B. dorsalis* samples from Asia and Africa studied by Qin *et al.* (2018).

p-distances *B. latifrons* captured in Italy

Minimum and maximum *p*-distances between the different COI fragments of Italian specimens and other conspecific homologous sequences downloaded from genetic databases are reported in table 2.

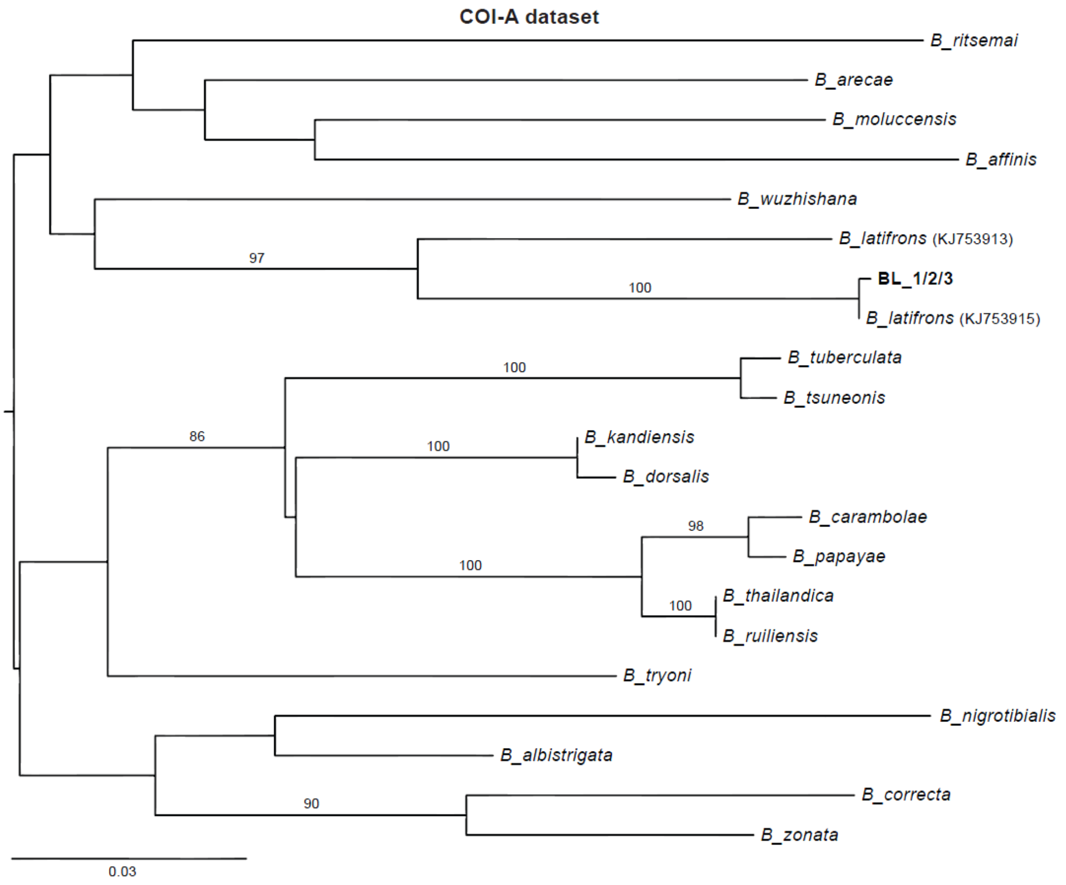
The highest distance 9.78% (± 1.138) (n = 104) was found among the sequences in the COI-A alignment.

Analysis of distances of COI-B portion highlighted a genetic distance of 0.62% (± 0.269) between BL_1 and BL_2 sequences, while the highest distance in this alignment (n = 16) resulted to be 1.85% (± 0.499).

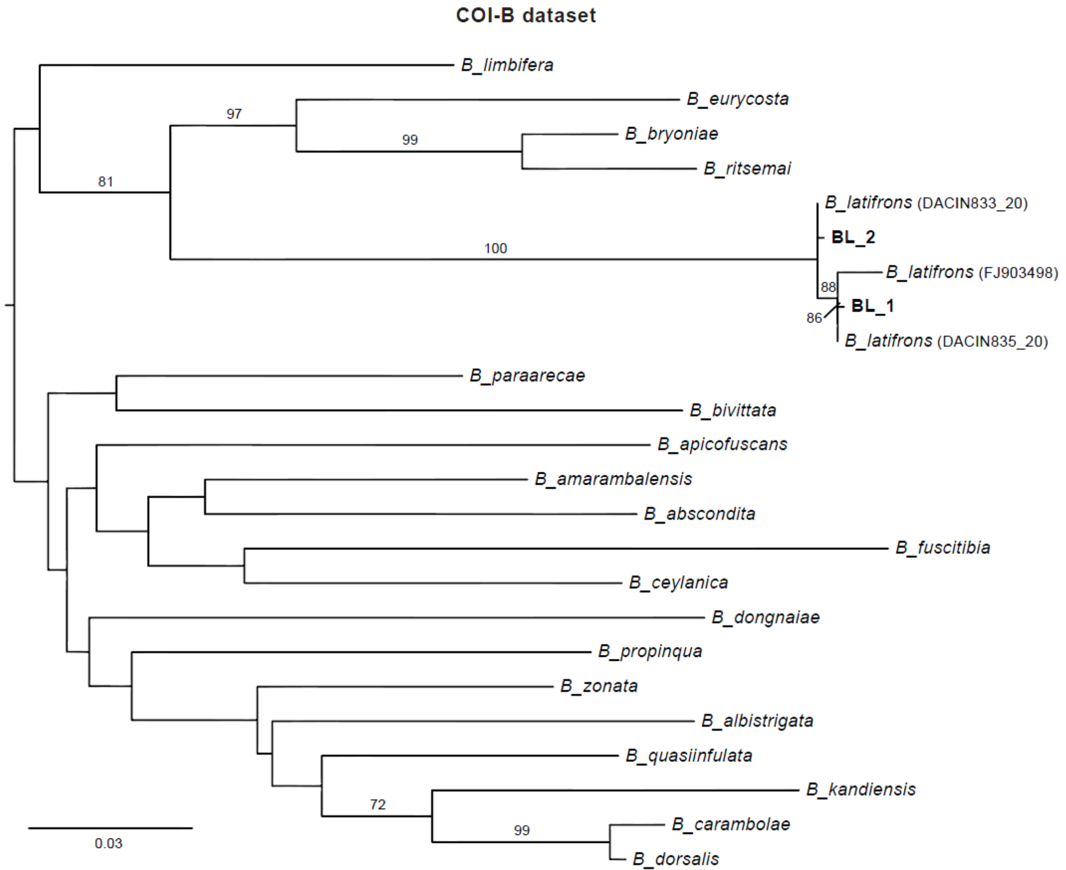
Distance analysis of COI-AB alignment (n = 15) showed the maximum value of 1.08% (± 0.249), differently, sequences of BL_1 and BL_2 showed a distance of 0.33% (± 0.158).

Table 2. Minimum and maximum *p*-distances between COI portions of Italian specimens and sequences available in genetic databases (GenBank-BOLD). D.I.N.: Database identification number.

COI portion	Code	Minimum *p*-distance		Maximum *p*-distance	
		% (±SE)	D.I.N.	% (± SE)	D.I.N.
COI_A	BL	0.15 (± 0.138)	KJ753915	7.81 (± 0.927)	KJ753913
COI_B	BL_1/BL_3	0.12 (± 0.111)	DACIN835-20	1.08 (± 0.392)	FJ903498
	BL_2	0.12 (± 0.12)	DACIN833-20	1.54 (± 0.473)	FJ903498
COI_AB	BL_1/BL_3	0.24 (± 0.124)	JX129505	1.07 (± 0.236)	KT881556
	BL_2	0.20 (±0.115)	DACIN836-20	0.87 (± 0.239)	KT881556



COI-A dataset



COI-B dataset

Figure 3. Representation of the relationship among *B. latifrons* samples found in Italy and other *Bactrocera* species based on portions A and B of COI region.

Discussion

The finding of *B. latifrons* represents the first detection in the field in Europe of this pest.

B. latifrons is considered native to India, Pakistan, Sri Lanka, Burma, China, Thailand, Laos, Vietnam, Malaysia, Taiwan, Japan (Liquido *et al*., 1994; Ishida, 2005; EPPO, 2020) but it invaded Hawaiian Islands since 1983 (Vargas and Nishida, 1985; Bokonon-Ganta *et al*., 2007) and Tanzania and Kenia since 2007 (Mwatawala *et al*., 2007; Mziray *et al*., 2010). Recently, *B. latifrons* has been reported as widespread in Burundi, where it attacks predominantly *Solanum aethiopicum* (Ndayizeye *et al*., 2019) while isolated outbreaks in California have been eradicated (EPPO, 2021c). In Europe, the species is absent, but it is often intercepted at entry points. In the last 15 years, 149 interceptions have been reported on Europhyt, and 86% of these were on *Capsicum* sp. (Europhyt, 2021). The risk of *B. latifrons* establishment outdoor is higher in Mediterranean Basin regions, Portugal, and the Black Sea coast (EPPO, 2020).

The first record of *B. latifrons* in Italy, during a monitoring plan aimed mainly at the detection of *B. dorsalis* individuals, may be considered as a serendipitous result, which, however, allows to express some important comments.

This work underlines the limit in the use of male lures or male specific attractants. ME is greatly effective on males of *B. dorsalis* and many other species, and it is undoubtedly effective to monitor these species in countries where they are present and widespread (Jang *et al*., 2017; Manoukisid *et al*., 2019) as well as where it is present in very low transient population (Nugnes *et al*., 2018). But, despite this, a monitory plan to early detect *Bactrocera* species in a new territory, if performed using only ME, presents gaps in the detection system. In fact, more than half of the Dacini is non-responsive to the male lures identified until now (McQuate *et al*., 2018). In contrast, protein baits like TY, even if less attractive than male lure on males, are suitable for detecting all the tephritid females (Epsky, 2014). Hence, during extensive monitory plans, the employing of both male lures and protein baits is preferable to optimize the resources deployed and to maximize results.

However, the control of numerous traps demands evaluations concerning the time and effort required. Indeed, collecting samples from traps baited with TY solution is time-consuming, just as the laboratory work required to process and analyse these samples is dramatically lengthy. Conversely, the time needed to process samples of ME baited traps is much shorter than the time required to process the TY baited traps. In the study area, indeed, the samples collected in ME baited traps consisted only of one or few specimens of the target pest or, eventually, of other species: *Bactrocera oleae* (Rossi), *Ceratitis capitata* (Wiedemann), *Rhagoletis completa* Cresson. Besides, TY needs to be replaced every 1-2 weeks, while ME can last 6-7 weeks (FAO/IAEA, 2018; Shelly *et al*., 2020).

One of the issues that emerged in this work is the total absence of captures of exotic fruit flies in 2020 in the monitored areas. Despite the use of many traps and the use of the specific attractant with Cade Oil and α-ionol, employed nearby and at the places of the previous captures, no *B. latifrons* specimens were caught. After two years of monitoring, with 7 captures of *B. dorsalis* in 2018 (Nugnes *et al*., 2018) and 7 in 2019 (4 *B. dorsalis* males and 3 *B. latifrons* females) (Vitiello *et al*., 2020 and present study), the total lack of captures in 2020, may be considered as a side effect of the global changes occurred in 2020. During this year, travellers and trade had to stop completely in Italy and almost worldwide due to the pandemic emergency. This new scenario probably led to a drastic reduction of pest accidental introductions and the result of zero exotic insect catches.

No captures occurred in 2020, so, for this reason, all the specimens belonging to *Bactrocera* genus detected in 2018 and 2019 have to be considered incursions. Hence, at present, the absence of stable populations of *B. latifrons* and *B. dorsalis* in Italy, at least regarding the monitored territories, is also corroborated by the finding of different haplotypes in different years.

The molecular analysis did not allow to discriminate the area of origin of the collected specimens. Indeed, COI-A sequence identical to Italian ones has been reported in different countries and continents, while, COI-B portion was shared by BL_1 and a Malaysian specimen (Yaakop *et al*., 2015). The absence of a complete COI sequence and correlated ITS1 sequence on databases cannot allow an estimation of the area of origin.

The COI-B portion is clearly more variable than COI-A, and shows differences even in the three collected specimens; thus, the COI-B portion (2183-3014) allows the more accurate identification of intraspecific haplotypes and more information in populations studies. Besides, the evaluation of distances in the COI portions (COI-AB and COI-B) highlighted minimum and maximum values that are congruent with the variability usually found in a single species.

On the opposite, the whole barcoding (COI-A) alignment showed high distance, that, even if unusual for the barcoding, is within the range of 11% of divergence found to be the average divergence in 16s and COI genes between *Bactrocera* species (Zhang *et al*., 2010). This value is probably due to the great amount of barcoding COI-A present in the most important databases (GenBank and Bold) that give us a larger picture of the high levels of variability of this species. Despite this, it could suggest the existence of a species-complex under the morphotype of *B. latifrons* as well as for *B. dorsalis* (Isaza *et al*., 2017).

Conclusions

The combination of traps and different types of attractants to monitor exotic fruit flies needs to be carefully assessed in wide monitory plans. The continuous monitoring of the territory, in order to early detect of new accidentally introduced fruit fly, is necessary, but requires the combined use of traps baited with male-lures and with food attractant as TY for females, as a powerful and preferable tool to optimize the monitory effort.

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